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TITLE: Acute Lung Injury Following Smoke Inhalation: Predictive Value of Sputum Biomarkers and Time Course of Lung Inflamation

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14. ABSTRACT

Background: The role of lung inflammatory mediators in the development of lung injury following smoke inhalation is unknown. Our objectives are to determine whether initial markers of inflammation or longitudinal changes in inflammatory markers are associated with ARDS or hypoxemia. Study design: Bronchial secretions from 200-250 intubated patients with smoke inhalation injury will be evaluated for initial and longitudinal changes concentrations of substance P, TNF-α, IL-1, IL-8, and IL-10, as well as cell count and differential every two hours to a maximum of 72 hours. Initial lung inflammation and changes in inflammatory markers will be compared in patients with and without subsequent significant lung injury. Progress to date: We have enrolled 123 subjects. To date, we have completed sample assays and data analysis on a subset of 21 subjects with early samples. We have assessed longitudinal changes in TNF-α, IL-1β, IL-8, IL-10, sFASl, substance P, IL-1RA, α2M, MMP-9, and TIMP-1 concentrations over the first 36 hours post-exposure, and looked at the relation between these biomarkers and hypoxemia and ARDS. We have defined temporal changes in IL-8, IL-1β, IL-1RA/IL-1β, and TNF-α/TNF-R2 post-exposure, and have found that initial concentrations of IL-8 and the ratio of α2-M to IL-8 are significant predictors of subsequent hypoxemia. Further analysis of additional markers, including neutrophils, proteases, and protease-inhibitors, have been started.

15. SUBJECT TERMS
smoke inhalation injury, burns, ARDS, inflammation

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INTRODUCTION

The goal of this research is to identify inflammatory mediators playing key roles in acute lung injury (ALI) following smoke exposure. Our objectives are to: 1) determine the value of initial concentrations of inflammatory mediators and their antagonists in predicting progression of ALI in smoke inhalation victims, and 2) to determine how these mediator concentrations change over time, which may also have predictive value and improve our understanding of the mechanism of smoke injury. We hypothesize that smoke inhalation results in rapid changes (within two hours) in lung inflammatory mediators, that initial changes in lung inflammatory mediators are predictive of the extent of subsequent lung injury, and changes over time in lung inflammatory mediators will precede clinical findings of acute lung injury. Over the past 5 years of this grant, we have evaluated initial concentrations and changes over time of inflammatory mediators in pulmonary secretions of approximately 21 ventilated patients suffering from smoke inhalation. The clinical course of these patients has been tracked, including % body surface area burn, days on a ventilator, days in ICU, pulmonary infiltrates, white blood cell count, fever, sputum volume, oxygen requirements, blood oxygenation, and development of ALI. In the next year, we hope to publish our current findings, and complete analyses and manuscripts on additional mediators.

There were no adverse events or complaints received in the course of this study.

BODY

There are two specific aims of this study: 1) To determine the predictive value of initial inflammatory markers in bronchial secretions of smoke inhalation victims for subsequent extent of lung injury; and 2) measure longitudinal changes in bronchial inflammatory mediators in smoke inhalation victims. The specific aims have been divided into five tasks as shown in the approved Statement of Work timetable (with the task description modified to clarify the meaning of each step).

	Year 1	Year 2	Year 3	Year 4
Recruitment/Enrollment	→→→→→→→→→→→→→→→→			
Tracheobronchial fluid sample collection	→→→→→→→→→→→→→→→→			
Medical outcome data collection	→→→→→→→→→→→→→→→→			
Sample Analysis		→→→→→→→→→→→→→→→→		
Data analysis/Manuscript preparation		→→→→→→→→→→→→→→→→		

The major activity of the first year of this research was to obtain Institutional Review Board (IRB) approval from the Army, the University of Arizona, and the Maricopa Integrated Health System (MIHS), which is the parent institution of the Arizona Burn Center where the subjects are enrolled in the study and the bronchial suction material and clinical outcome data collected. This process took much longer than anticipated and therefore required shifting the start of all of the timetable tasks into year 2. We therefore continued subject recruitment and sample collection through year 4. In the past year, we have completed acquisition of data, data entry, processing of laboratory samples, data analysis, and manuscript preparation. The total number of patients consented is 112 and we received a “waiver of consent and HIPAA authorization” for an

additional 11 patients who met the inclusion criteria but expired before they or family members could be consented, for a total study population of 123 subjects. Of these, 93 are male, 30 female. No ethnicity data was collected on the population. Subject age ranged from 2-88 years, with a mean of 38 years (Table 1). Clinical outcome data has been collected on 122 of the 123 consented subjects. Among these patients, there have been 21 deaths (17.2%). Organ failure was recorded in 49% of subjects, sepsis in 41%, and trauma in 10%. A quarter of the subjects had greater than 30% total body surface area full thickness burns.

Table 1: Characteristics of subjects (enrolled June 2003 -April 2006).

Mean age (range)	38.4 yrs (1.8 - 88)
Male	93/123 (75.6%)
Total body surface burn	
0%	44/120 (36.7)
1-15%	22/120 (18.3%)
16-35%	31/120 (25.8%)
>35%	23/120 (19.2%)
Organ failure	54/109 (49.5%)
Trauma	11/109 (10.1%)
Fracture	8/109 (7.3%)
Died within 72 hrs	21/119 (17.6%)

Arterial blood gas data, including PaO_2, FIO_2, and positive end expiratory pressures (PEEP), were collected on all but 5 subjects, generally starting within 6 hrs of smoke exposure and continuing for 72 hrs post-intubation.

Tracheobronchial samples were collected at approximately two hour intervals, starting within 6 hours of intubation for over 90% of subjects. Laboratory assays on the tracheobronchial samples have required considerable experimentation with different preservation techniques and different types of assays. To date, we have collected and processed over 2958 samples on a total of 123 subjects, and incorporated time of sample collection for all patients into our database.

We have completed assays of interleukin (IL)-1 beta, IL-8, and tumor necrosis factor (TNF)-alpha (using the R&D Systems Quanti-Glo Elisa Kits) for 32 subjects. High, medium and low controls were added to the testing protocol for all assays after 11/15/04 to ensure accuracy of the testing protocol. Both soluble Fas Ligand (sFASL) and Transforming Growth Factor-beta 1 (TGF-β1) were measured on 21 subjects using the R&D Systems Quantikine Elisa Kits, after verification of the testing procedure. Measurement of protein concentration using the Sigma BCA-1 Protein Determination Reagent Kit was completed after kit verification for use on sputum and bronchial lavage samples. Urea nitrogen was assessed using the Pointe Scientific, Inc., Reagent Set # B7550-400 after the methodology was verified using the protocol for verification of new tests. Urea levels in the tracheobronchial secretions were consistently low. Additional biomarkers were assayed on 21 subjects for whom we had tracheobronchial samples within the first 6.5 hours and arterial blood gas measures between 6 and 72 hours. These markers included: IL-1 Receptor Antagonist (IL-1RA), tissue inhibitor of metalloproteinase-1

(TIMP-1), and matrix metalloproteinase-9 (MMP-9) (using R&D Systems Quantikine Kits), and alpha-2 macroglobulin (α2M) (using ALPCO Diagnostics Kits with in-house controls).

Cell counts and differentials were completed on 93 subjects. The initial procedure called for preservation of the sample in methanol. Because of the unavoidable time delay between collection and processing, methanol did not preserve the cells sufficiently for accuracy for either the cells counts or the differentials. 40% Glycerol was used with better cell counting results, but cells were too degenerated upon storage to accurately perform the differential. Cytolyte was then used yielding cells that displayed less cellular disintegration; however, the cells contracted, which made cell counting difficult and the staining characteristics for the differential were unreliable. Histochoice was then used which showed much better results for cell counting and differentiation and is the method of preservation used at this time.

Substance-P was assayed on 16 subjects using the R&D Elisa Assay Kit. Results were consistently under the detection limit. After conferring with company representatives and laboratorians experienced in testing Substance-P levels, an additional preliminary concentration/purification protocol using a C-18 reverse phase cartridge (Sep-Pak) was performed on several subjects. Repeated Substance-P analyses remained under the detection limits in our tracheobronchial samples, so these assays were discontinued.

We have submitted a manuscript entitled, "Tracheobronchial Markers of Lung Injury in Smoke Inhalation Victims" to the journal *Chest*, and are working on a second manuscript, "Predictive Value of Tracheobronchal Cytokine and Protease Inhibitors in Smoke Inhalation Injury." In these analyses, we found that the ratio of PaO_2:FIO_2, a measure of oxygenation, decreased with time since exposure ($p<0.0001$) (Figure 1). Approximately 66% of patients developed PaO_2/FIO_2

Figure 1. PaO_2/FIO_2 ratios are inversely related to time since exposure (p=0.001 in a random coefficients model).

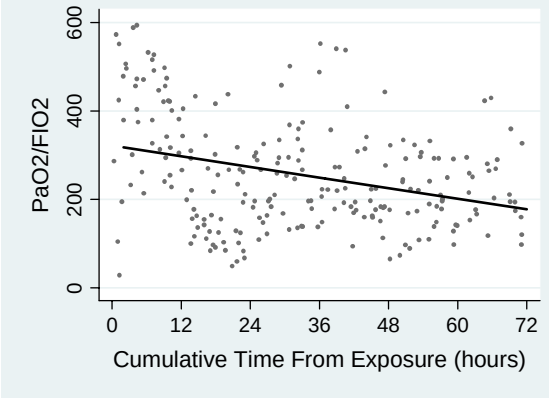


Table 2. Characteristics of subjects with tracheobronchial samples collected within 6.5 hours of intubation (total n= 21).

Mean age (range)	40.8 yrs (12.6-70.1)
Male	20 (95%)
Total body surface area burn	
<15%	8 (38.1%)
15-35%	4 (19.0%)
>35%	9 (42.9%)
Fracture and/or trauma	5 (23.8%)
$PaO_2/FIO_2 \leq 200$ before 72 hrs	14 (66.7%)
ARDS	9 (42.9%)
Maximum PEEP setting	
5-9	8 (38.1%)
10-14	11 (52.4%)
≥ 15	2 (9.5%)
Died within 72 hrs	1 (4.8%)

<200 (acute hypoxemia) within 72 hrs of exposure, and 43% of a subgroup of 21 with tracheobronchial biomarkers measured within 6.5 hrs of intubation developed ARDS (Table 2).

We also found a sharp increase in IL-1β and IL-8 in the first six hours post-exposure ($p<0.001$) (Fig 2), but no significant temporal trends in IL-10, TNF-α, TGF-β, sFasL or C5a (Fig 3). Initial IL-8 concentrations were significantly lower in subjects who had a minimum PaO_2/FIO_2 of ≤ 200 with PEEP <6, than in subjects whose minimum ratio was >200 ($p=0.03$), but initial concentrations of none of the other biomarkers measured showed any relation to development of ARDS or acute hypoxemia (Table 3).

Figure 2 . Mean levels of IL-8, IL-1β, and TNF-α in tracheobronchial fluid samples over time since intubation.

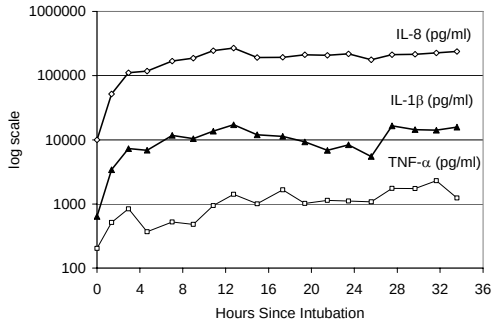


Figure 3. Mean levels of TGF-β1, sFasL, IL-10 and C5a.

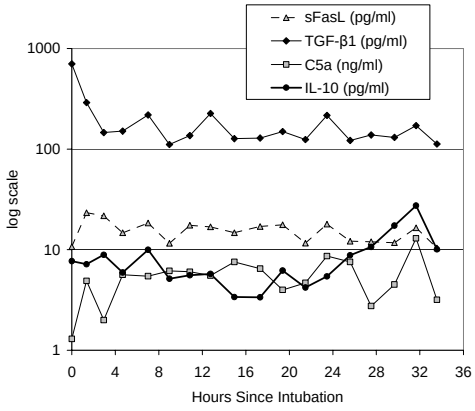
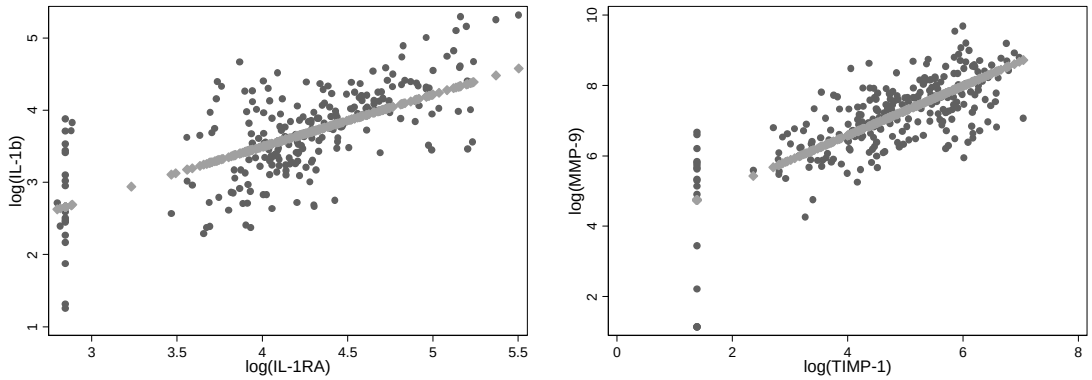


Table 3. Random coefficients models predicting PaO_2/FIO_2 over 72 hours, adjusted for initial log concentration of IL-8 (covariance matrix pattern for random effects unstructured).

	Estimate	**SE**	**p-value**
Fixed-effects Parameters			
intercept	132.57	81.65	0.104
cumulative hours	-1.94	0.57	0.001
(log) initial IL-8	47.83	19.17	0.013
Random–effects Parameters			
sd (intercept)	88.61	22.39	
sd (slope)	2.00	0.58	
correlation (intercept, slope)	-0.85	0.11	
sd (residual)	99.93	5.11	
Log likelihood	-1416.7		

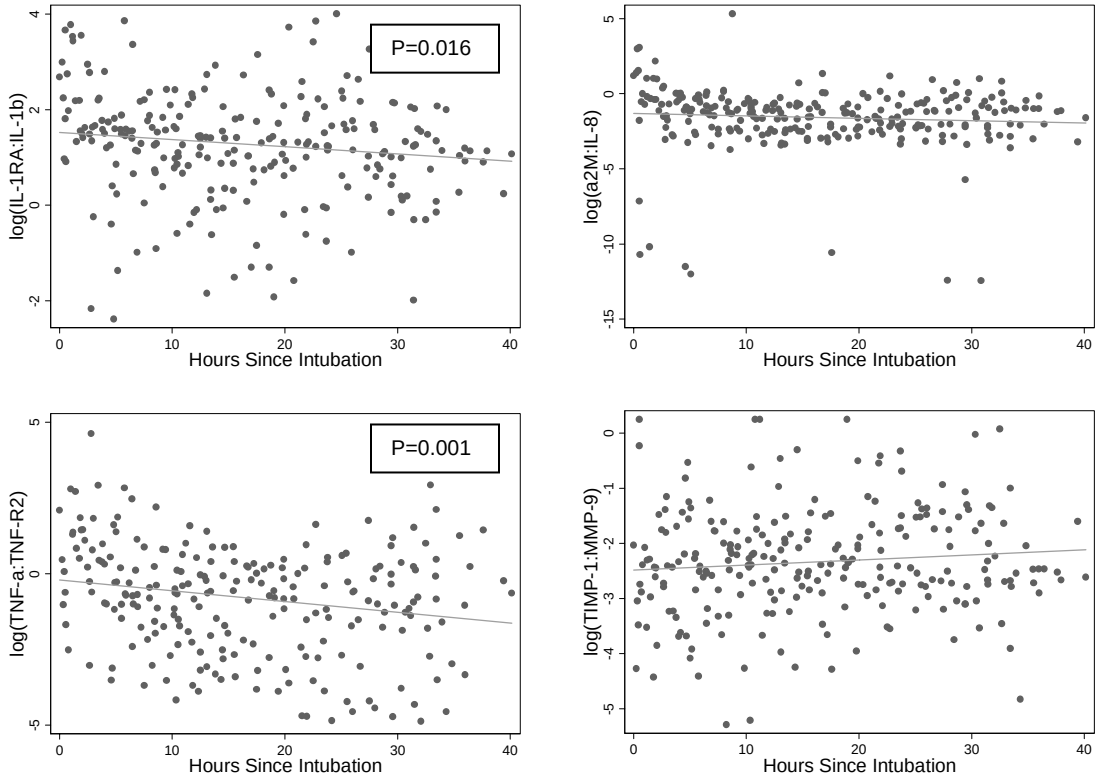
We used a random effects model to test the relationship between cytokines and their antagonists at multiple time points. The log values of IL-1β and IL-1RA were positively correlated (β=0.722, <0.001), as were the log values of TIMP-1 and MMP-9 (β=0.703, p<001) (Figure 4), TNF-R2 and TNF-α (β=0.218, p<0.001), and α2-M and IL-8 (β=0.095, p<0.001) (not shown).

Figure 4. Correlation of log values of IL-1β and IL-1RA and MMP-9 and TIMP-1 at multiple time points in 21 subjects.



Both the log of the ratios of TNF-α/TNF-R2 and IL-1RA/IL-1β decreased with time since intubation in random coefficients models that take into account repeated measures on each subject (p<0.001, p=0.016, respectively). Neither the log of α2-M/IL-8 nor the log of MMP-9/TIMP-1 showed a trend with time (Figure 5).

Figure 5. Relation of log-transformed biomarker ratios to time since intubation.



The only biomarker ratio that was predictive of PaO2/FiO2 in random coefficient models was the log of α2-M:IL-8. After adjustment for cumulative time since exposure, the ratio declined with increasing oxygenation (p=0.029) (Table 3).

Table 3. Random coefficient model with PaO_2/FIO_2 as a dependent variable and the log of α1-M/IL-8 and time since exposure.

PaO2/FIO2	Coefficient	Std. Err.	z	P>|z|	[95% Conf.	Interval]
Hours since exp	**-1.768087**	**.7130401**	**-2.48**	**0.013**	**-3.16562**	**-.3705545**
Log(a2m/il8)	**-8.194132**	**3.761403**	**-2.18**	**0.029**	**-15.56635**	**-.8219181**
intercept	299.2057	23.70845	12.62	0.000	252.738	345.6734

Random-effects Parameters	**Estimate**	**Std. Err.**	**[95% Conf.**	**Interval]**
Subject ID: unstructured				
sd(hours since exposure)	2.354858	.723008	1.290095	4.298408
sd(intercept)	72.61485	24.01314	37.97869	138.8388
corr(hours,intercept)	-.806411	.1556858	-.9632601	-.239375
sd(Residual)	100.9226	6.096443	89.65401	113.6075

LR test vs. linear regression: chi2(3) = 19.47 Prob > chi2 = 0.0002

KEY RESEARCH ACCOMPLISHMENTS

- In our population of smoke inhalation victims, subjects almost uniformly manifest a decline in their PaO_2/FIO_2 ratio to below 300, which is consistent with development of acute lung injury.
- We have demonstrated that bronchial suction material can be used for longitudinal analysis of cytokines using commercially available ELISA kits.
- IL-1β and IL-8 show a steep increase in the first six-seven hours post-exposure.
- Initial IL-8 concentrations are significantly lower in patients with minimum PaO_2/FIO_2 ratios above 200.
- The log transformed ratios of IL-1RA/IL-1β and of TNF-α/TNF-R2 decrease with time from exposure.
- After adjustment for time since exposure, the ratio of α2-M/IL-8 decreases with improved PaO_2/FIO_2 ratios.
- We have begun to look at protease inhibitors, including alpha-1 antitrypsin and secretory leukocyte protease inhibitor (SLPI) to assess potentially protective effects against lung injury.

REPORTABLE OUTCOMES

1) Abstract and poster: May 25, 2004
American Thoracic Society 100th International Conference, Orlando, FL
"Longitudinal changes in tracheobronchal suction fluid inflammatory mediators following smoke Inhalation."

2) Abstract and poster: May 23, 2005
American Thoracic Society 101st International Conference, San Diego, CA
"Use of tracheobronchial suctionate inflammatory markers to predict subsequent lung injuring in smoke inhalation victims"

3) Abstract and presentation:
North American Congress of Clinical Toxicology (NACCT) Sept 9-14, 2005
"Longitudinal changes and tracheobronchial biomarkers of acute lung injury"

4) Abstract submitted to *North American Congress of Clinical Toxicology (NACCT)* April 1, 2007
"Ratios of tracheobronchial pro- and anti-inflammatory mediators in smoke inhalation victims."

5) Journal article submitted to: *Chest* May 9, 2007
"Tracheobronchial markers of lung injury in smoke inhalation victims."
Margaret Kurzius-Spencer, MS, MPH; Kevin Foster, MD; Sally Littau, BS; Karen J. Richey, RN; Beth M. Clark, BS[2]; Duane Sherrill, PhD; Richard B. Goodman, MD; Scott Boitano, PhD; Jefferey L. Burgess, MD, MPH

6) Manuscript in preparation: present
"Predictive value of tracheobronchial cytokine and protease inhibitors in smoke inhalation injury."

CONCLUSIONS

Smoke inhalation injury continues to cause significant morbidity and even mortality, as demonstrated by the clinical outcomes of our subjects to date. No diagnostic test or specific pharmaceutical therapy is available for acute lung injury following smoke exposure. We have shown that longitudinal evaluation of tracheobronchial suctionate from smoke inhalation victims can be analyzed for measurement of inflammatory mediators. If we show that specific inflammatory mediators secreted in the lungs in the first two-six hours following smoke exposure are predictive of later decline in PaO_2/FIO_2 ratio, then it will be reasonable to consider evaluation in animal models and, if successful, in human clinical trials, of pharmacological agents working through antagonism or promotion of the effects of these mediators.

Future directions include the analysis of additional inflammatory mediators and measurement of these inflammatory mediators in small animal models of smoke exposure. Thus far, we have found a rapid increase in tracheobronchial fluid of both IL-1β and IL-8 in the first six hours post-intubation, an increase

in percent neutrophils within 12 hours of smoke exposure, and a steady decrease in the ratios of IL-1RA/IL-1β and TNF-α/TNF-R2 over 36 hours. Contrary to expectation, we found that initial IL-8 concentrations were lower in subjects who later developed more severe lung injury, and a similar trend was found with IL-1β. A possible explanation may be that in patients with severe smoke exposure, there are modifications to or loss of IL-8. In the next year, we plan to analyze the tracheobronchial concentration of alpha 2-macroglobulin (α2-M) in all subjects with early samples. A2-M is a measure of altered lung permeability and may give us an early measure of severity of exposure. A2-M will give us an intermediate endpoint which can be related both to initial cytokine concentrations as well as clinical endpoints.

In addition, we are completing analysis of additional inflammatory mediators (IL-1RA, TNF-R2, MMP-9, TIMP-1, AAT, and SLPI) that may be predictive of the development of ALI, alone or as ratios, and also could potentially serve as therapeutic targets. As mentioned in previous reports, the selection of additional inflammatory mediators will be based in part on the availability of potential therapeutic interventions associated with the selected mediators.

REFERENCES

Please see Appendix 3 for documentation of literature review. There have been no recently published articles that would alter our protocols.

APPENDICES

1) Abstract, *American Thoracic Society 100th International Conference, May 25, 2004, Orlando, FL.*
"Longitudinal changes in tracheobronchal suction fluid inflammatory mediators following smoke inhalation"

2) Abstract, *American Thoracic Society 101st International Conference,May 23, 2005, San Diego, CA.*
"Use of tracheobronchial suctionate inflammatory markers to predict subsequent lung injuring in smoke inhalation victims"

3) Abstract and presentation:
North American Congress of Clinical Toxicology (NACCT), Sept 9-14, 2005.
"Longitudinal changes and tracheobronchial biomarkers of acute lung injury"

4) Abstract submitted to *North American Congress of Clinical Toxicology (NACCT), April 1, 2007.*
"Ratios of tracheobronchial pro- and anti-inflammatory mediators in smoke inhalation victims."

5) Paper submitted to: *Chest* *May 9, 2007*
"Tracheobronchial markers of lung injury in smoke inhalation victims"

Margaret Kurzius-Spencer, MS, MPH; Kevin Foster, MD; Sally Littau, BS; Karen J. Richey, RN; Beth M. Clark, BS[2]; Duane Sherrill, PhD; Richard B. Goodman, MD; Scott Boitano, PhD; Jefferey L. Burgess, MD, MPH

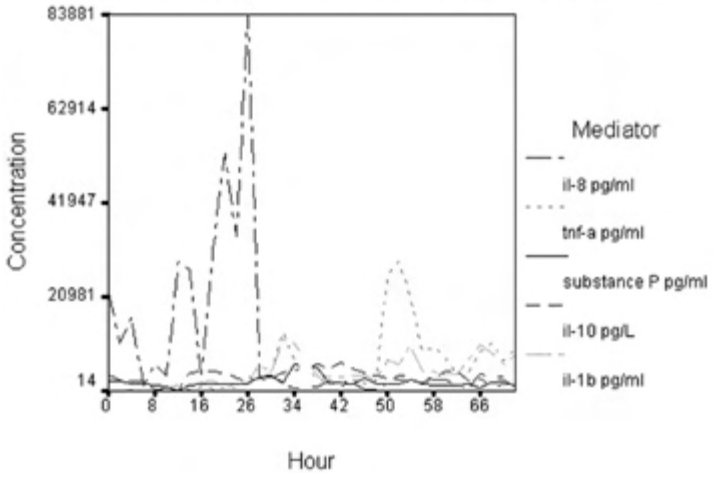
Appendix 1.

[C52] [Poster: E14] Longitudinal Changes in Tracheobronchal Suction Fluid Inflammatory Mediators Following Smoke Inhalation

J.L. Burgess, A. Josyula, K.N. Foster, T.A. Hysong, N.S. Francis University of Arizona, Tucson, AZ; Maricopa Integrated Health System, Phoenix, AZ

Rationale: The role of inflammatory mediators in the development of human smoke inhalation injury is not well understood. We hypothesize that initial changes in lung inflammatory mediators are predictive of the extent of subsequent lung injury. *Methods*: As a first step in investigating this process, mediator concentrations in tracheobronchal secretions of ventilated patients were collected every two hours over the first 72 hours following smoke inhalation. Sample supernatants were analyzed by ELISA. *Results*: For the first four subjects for which samples have been analyzed, comparing the initial mediator concentration with the peak level gave the following fold increases: interleukin (IL)-1β 70-106; IL-8 6-115; IL-10 1-7; TNF-α 225-2560; and substance P 1-4. The longitudinal changes in one of the subjects enrolled in the study are illustrated in Figure 1. *Conclusions*: Longitudinal collection of tracheobronchal suction material provides a means of measuring changes in inflammatory mediators which will be evaluated for association with development of acute lung injury.

Figure 1. Longitudinal changes in inflammatory mediator concentrations in a single subject



Tuesday, May 25, 2004 8:15 AM

Appendix 2.

[**] Thematic Poster Session (Abstract Page: A641) Session:

8:15 am-4:15 pm, ENVIRONMENTAL AND OCCUPATIONAL PULMONARY TOXICOLOGY

American Thoracic Society : Abstract # 956596

Title: **Use of tracheobronchial suctionate inflammatory markers to predict subsequent lung injury in smoke inhalation victims.**

J.L. Burgess, MD, MPH [1], K.N. Foster, MD [2], S.R. Littau [1], M. Kurzius-Spencer, MS, MPH [1], K.J. Richey, RN [2], A.B. Josyula, MD, MPH [1] and R.M. Shipitalo [1].
[1] University of Arizona, Tucson, AZ
[2] Arizona Burn Center, Maricopa Integrated Health System, Phoenix, AZ.

Rationale: Smoke inhalation victims are at high risk of developing acute respiratory distress syndrome (ARDS). Given the delay of 12 or more hours from exposure to development of ARDS, a prognostic test applied early in the clinical course could potentially identify patients for whom specific interventions may be of value. *Methods*: Patients with inhalation injury admitted to a regional burn center and requiring intubation were eligible for the study. Tracheobronchial suction fluid was collected every two hours. Sample supernatants were analyzed for interleukin-1, -8, (IL-1, IL-8) and tumor necrosis factor alpha (TNF-) by ELISA. Medical history and clinical course including arterial oxygenation (PaO2) and fraction of inspired oxygen (FIO2) were collected. *Results*: Mean PaO2/FiO2 decreased over time, generally reaching its nadir at 18-28 hours post-intubation. Regression models were run to assess the relationship between early IL-1, IL-8 and TNF- concentrations and subsequent PaO2/FiO2 measurements, adjusting for potential confounders including age, asthma, COPD, percent of full thickness body surface burned, and fractures suffered. In an analysis of eight patients with complete information, the log of IL-1 from a bronchial sample at 4 hours post-intubation (p=.008), age (p=.023), and % body surface burned (p=.022) were all significant predictors of PaO2/FiO2 at 18 hours. At similar time points, tracheobronchial TNF-, but not IL-8, was predictive of later PaO2/FiO2. *Conclusion*: In patients admitted to a burn center with smoke inhalation requiring intubation, IL-1 concentrations in tracheobronchial suction material at four hours were predictive of PaO2/FIO2 at 18 hours after exposure.

This research was supported by the U.S. Army Peer Review Medical Research Program, grant DAMD17-02-1-0673.

Appendix 3.

North American Congress of Clinical toxicology (NACCT) Abstract 4/21/05

Title: **LONGITUDINAL CHANGES AND TRACHEOBRONCHIAL BIOMARKERS OF ACUTE LUNG INJURY**

Background/Objectives: Smoke Inhalation victims are at high risk of lung inflammation and acute lung injury. A prognostic test applied early in the clinical course could potentially be used to identify patients for whom specific interventions might be of value.

Methods: Patients with inhalation injury admitted to a regional burn center and requiring intubation were eligible for the study. Tracheobronchial suction fluid was collected every two hours and supernatants were analyzed for interleukins (IL) -1b and -8, and tumor necrosis factor alpha (TNF-a) by ELISA. Data on clinical course included arterial oxygenation (PaO2) and fraction of inspired oxygen (FIO2) at 2-4 hour intervals. Standard parametric and non-parametric statistics were used.

Results: Of 56 subjects, 6 (10.7%) died, and over 53% of surviving subjects were diagnosed with ARDS. PaO2/FiO2 decreased over time ($p<0.0001$), generally reaching its nadir about 20-30 hours post-intubation. Log-transformed cytokine values were significantly correlated (all $p<0.001$) and increased significantly over time (all $p<0.001$), but most sharply in the first four hours post-intubation. PaO2/FiO2 had a significant inverse relation to IL-1b and TNF-a ($p<0.025$ and $p<0.035$, respectively). Log IL-8 values in the first 6 hours were significantly higher in patients with sepsis ($p<0.023$). Although early cytokines tended to be higher in the presence of trauma, fracture or percent full thickness burn, the differences were not significant.

Conclusion: In patients admitted to a burn center with smoke inhalation requiring intubation, PaO2/FiO2 decreased over time and IL-1b, -8, and TNF-a concentrations in tracheobronchial suction material increased markedly in the first four hours. Cytokines measured in the first 6 hours post-intubation were not predictive of PaO2/FiO2.

Funded By: This research was supported by the U.S. Army Peer Review Medical Research Program, grant DAMD17-02-1-0673.

Appendix 4.

North American Congress of Clinical toxicology (NACCT) Abstract April 1, 2007

RATIOS OF TRACHEOBRONCHIAL PRO- AND ANTI-INFLAMMATORY MEDIATORS IN SMOKE INHALATION VICTIMS

ABSTRACT

Background: Smoke inhalation is a major cause of acute respiratory failure in patients admitted to burn centers. Early changes in the balance between pro- and anti-inflammatory mediators have not been studied in this group, and may suggest opportunities for early intervention.
Objectives: To assess early longitudinal changes in the ratios of tracheobronchial fluid anti- and pro-inflammatory mediators and determine their utility as predictors of subsequent lung injury.
Methods: Partial pressure of arterial oxygen (PaO_2) and the fraction of inspired oxygen (FIO_2) were recorded approximately every six hours from intubated smoke inhalation patients admitted to a regional burn center, and tracheobronchial suction fluid was collected every two hours. Suctionate was assayed for the following mediators and their complements: interleukin-1β and interleukin-1 receptor antagonist (IL-1β:IL-1ra), IL-8 and alpha-2 macroglobulin (IL-8:α2-M), tumor necrosis factor-alpha and soluble TNF receptor 2 (TNF-α:TNFR2), and matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 1 (MMP-9:TIMP-1). Temporal trends in marker ratios in the first 36 hours after exposure, and the relation between the earliest complement ratios and measure of oxygenation (PaO_2/FIO_2) during the first 72 hours were assessed using random coefficients modeling and cross-sectional analysis.
Results: In 21 subjects with tracheobronchial samples collected within 6.5 hrs of intubation, 14 (66.7%) developed acute hypoxemia ($PaO_2/FIO_2 \leq 200$) within 72 hrs of exposure. Levels of each mediator and its complement were positively correlated. Only the log of the ratio of α2-M and IL-8 is a negative predictor of PaO2/FIO2 after adjustment for cumulative time since exposure (p=0.029).
Conclusions: Lower levels of α2-M and α2-M in relation to IL-8 concentrations are associated with improved PaO2/FIO2 ratios.

Appendix 5.

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TRACHEOBRONCHIAL MARKERS OF LUNG INJURY IN SMOKE INHALATION VICTIMS

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ABSTRACT

Background: Although smoke inhalation injury victims frequently develop acute respiratory distress syndrome (ARDS), no early prognostic tests are currently available. Our objectives were to determine early longitudinal changes in tracheobronchial fluid inflammatory markers and assess the value of initial concentrations as predictors of subsequent lung injury.

Methods: Partial pressure of arterial oxygen (PaO_2) and the fraction of inspired oxygen (FIO_2) were recorded approximately every six hours from intubated smoke inhalation victims admitted to a regional burn center. Tracheobronchial suction fluid was collected every two hours and assayed for interleukins (IL-1β, -8, and -10), tumor necrosis factor-alpha (TNF-α), transforming growth factor beta (TGF-β1), soluble Fas ligand (sFasL), and complement factor 5a (C5a). Temporal trends in marker concentrations over 36 hours and the relations between initial concentrations and lowest PaO_2/FIO_2 or ARDS within 72 hours were assessed using random coefficients modeling and cross-sectional analysis.

Results: In 21 subjects with tracheobronchial samples collected within 6.5 hrs of intubation, 14 (66.7%) developed acute hypoxemia ($PaO_2/FIO_2 \leq 200$) within 72 hrs of exposure and 9 (42.9%) developed ARDS, as defined by the American-European consensus conference on ARDS. IL-8 increased sharply in the first 6.5 hours post-exposure ($p<0.001$), and IL-1β in the first 6.1 hours ($p<0.001$). No significant temporal trends in IL-10, TNF-α, TGF-β1, sFasL or C5a were found. Only initial IL-8 was associated with increased PaO_2/FIO_2 ($p=0.013$) and with a minimum $PaO_2/FIO_2 > 200$ ($p=0.042$) over 72 hours.

Conclusions: In smoke inhalation victims, tracheobronchial IL-1β and IL-8 increase rapidly and high initial IL-8 may predict improved oxygenation.

Keywords: smoke inhalation injury, burns, ARDS, interleukin-8, interleukin-1 beta.

Abbreviations: ARDS = acute respiratory distress syndrome; ALI = acute lung injury; PaO_2 = partial pressure of arterial oxygen; FIO_2 = fraction of inspired oxygen; PEEP = positive end-expiratory pressure; IL= interleukin; TNF = tumor necrosis factor; TGF = transforming growth factor; sFasL = soluble Fas ligand; C5a = complement factor 5a; DTT = dithiothreitol; BDL = below the limit of detection; RCM = random coefficients model.

INTRODUCTION

In the U.S., there are more than one million burn injuries annually.[1,2] Respiratory complications are the major cause of mortality from smoke inhalation and burns, and early consequences commonly include increased alveolar permeability, acute pulmonary edema, and an accumulation of both pro- and anti-inflammatory cytokines,[3,4] all hallmarks of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Criteria for diagnosing ALI and predicting progression to ARDS have poor reliability in part due to the heterogeneity of the patient population and underlying causes.[5]

Specific risk factors for ARDS due to smoke inhalation injury include duration and dose of smoke,[6] age,[7] extent of skin burn,[2] and chronic lung and liver disease.[8] However, indices of oxygenation and ventilation and lung injury scores are not predictive of mortality risk at the time of diagnosis.[8] At present, there is no diagnostic test available that could be applied early in the clinical course to predict the extent of subsequent lung injury. In addition, no specific therapy is available for smoke inhalation injury other than supportive care.

Evaluation of inflammatory markers in the lung rather than in the blood is likely to provide the best measure of lung inflammation.[9] Measurable changes of markers in lung lumenal fluids during epithelial compromise and inflammatory cell recruitment could lend to the development of prognostic and/or diagnostic tests. Since release of these inflammatory markers by macrophages and epithelial cells increases the emigration of neutrophils into the lung and their concomitant activation, antagonism of pro-inflammatory markers could also prove a therapeutic mechanism. Lung inflammation alters alveolar permeability, with resultant entry of water and blood proteins into the alveoli and the development of pathology associated with lung injury.[4,2]

In this study, we measured secreted inflammatory markers associated with smoke exposure, ARDS, or epithelial cell injury during the initial 72 hours following exposure, when ALI and/or ARDS could potentially be limited or prevented. Our hypothesis was that early increases in proinflammatory markers would be predictive of the extent of lung injury, as measured by the lowest ratio of partial pressure of arterial oxygen (PaO_2) over the fraction of inspired oxygen (FIO_2). Our study objectives were to measure

longitudinal changes in bronchial inflammatory markers and to determine the predictive value of initial inflammatory markers in bronchial secretions for subsequent extent of lung injury in smoke inhalation victims.

METHODS

Subjects and Data Collection

This study was approved by the Institutional Review Boards of the University of Arizona, Tucson, AZ, and Maricopa Integrated Health System, Phoenix, AZ. All patients admitted to the Arizona Burn Center between June 2003 and April 2005 who had suffered smoke inhalation and required intubation were eligible for participation in this study. There were no exclusion criteria. However, only the first 21 subjects with initial tracheobronchial samples collected within 6.5 hours of smoke exposure are included in this analysis. Consent was obtained from family members of the patients. Information on medical history, trauma and percent body surface area burn was collected on admission. PaO_2, FIO_2, and positive end-expiratory pressure (PEEP) settings were recorded on average every six hours over a duration of 72 hours. A radiologist blinded to the subjects' outcomes read chest radiographs for presence or absence of bilateral infiltrates consistent with ARDS. The definition of ARDS used in this study followed the American-European consensus conference on ARDS: presence of acute hypoxemia with a PaO_2/FIO_2 of 200 mmHg or less, bilateral infiltrates seen on frontal chest radiograph consistent with pulmonary edema, and no clinical evidence of left atrial hypertension.[10]

Tracheobronchial Suctionate Protocol

As part of routine pulmonary care, tracheobronchial suction fluid was collected at approximately two-hour intervals for up to 36 hours. The respiratory technician instilled normal saline (15 ml in subjects over 15 years and 10 ml in patients 2-15 years) through the endotracheal tube, and then used a suction catheter with trap (Lukens, Sherwood Medical, Albuquerque, NM) to aspirate as much fluid as possible. A portion of the sample was mixed 1:1 with fixative (Histochoice MB, Amresco, Solon, OH) and kept at 2-8° C prior to cytocentrifugation and cell count. The remainder of the sample was frozen at -80° C. After transporting the sample to the University of Arizona, the suctionate was thawed and the sample volume was combined with an equal volume of 0.1% dithiothreitol (DTT) solution (Sputolysin;

Calbiochem, San Diego, CA). Samples were vortex mixed and then centrifuged at 2400 RPM for 16 minutes at 4° C. The supernatant was frozen at -80^{o} C until time of cytokine analysis.

Laboratory Procedures

Levels of interleukin (IL) -1 beta (IL-1β), IL-8, and tumor necrosis factor-alpha (TNF-α) were measured over the first 36 hours post-smoke exposure using R&D Systems (Minneapolis, MN) Quanti-Glo ELISA and IL-10 using a R&D Systems High Sensitivity Quantikine kit. Soluble Fas ligand (sFasL) and transforming growth factor beta (TGF-β1) were measured using R&D Systems Quantikine ELISA kits. Levels of Complement 5a (C5a) were measured using the BD Biosciences Human C5a ELISA kit (BD Biosciences Pharmingen, San Diego, CA). Protein concentration was measured using the Sigma BCA-1 Protein Determination Reagent Kit (Lincoln Park, MI) and measurement of urea nitrogen was performed using the Pointe Scientific, Inc., Reagent Set (St. Louis, MO).

Statistical Analysis

Marker concentrations of IL-1β, IL-8, and TNF-α were normalized using $\log_{10}$-transformation. Fewer than 1% of samples were below the limit of detection (BDL), and these were assigned a value of one-half the detection limit. IL-10, TGF-β1, C5a, and sFasL concentrations were divided into tertiles for analyses, as over 25% of the samples were BDL. T-tests and Mann-Whitney rank-sum tests were used to compare initial mean or ranks of markers, respectively, by clinical outcome. Ordinal (3 category) variables were created for both PEEP setting and percent total body surface burn. To assess associations between categorical variables, Fisher's exact test was used. Multiple logistic regression models were used to look at the risk of initial marker concentrations on binary outcome variables (ARDS/noARDS, $PaO_2/FIO_2 \leq 200/PaO_2/FIO_2>200$). Longitudinal, temporal relations between measures of PaO_2/FIO_2 and cumulative time since exposure and between markers and time since exposure were evaluated using random coefficients models (RCM). We assumed the covariance matrix of random effects between the

random intercept and slope was unstructured, and nested RCM models were compared using the likelihood-ratio test. Breakpoint analysis[11] was used to fit two straight lines constrained to join at a common point in the RCM model of IL-8 and IL-1β over time. Tests of significance were all two-sided and a critical value of α=0.05 was used. Stata 9.2 (StataCorp LP, College Station, TX) and SPSS 14.0 (SPSS Inc., Chicago, IL) were used for all statistical analyses.

RESULTS

Study population

Demographic, medical history and exposure data collected on 21 subjects with initial measurement of tracheobronchial marker concentrations within 6.5 hours of intubation are presented in Table 1. The study population was predominantly male and ranged from 12-70 years of age. Over 42% of subjects suffered total body surface area burns on more than 35% of their body. Subjects with greater than 35% total body surface area burn were more likely to develop ARDS (p=0.018) within 72 hours, compared with subjects with less extensive burns. Two-thirds of the subjects had a PaO_2/FIO_2 of 200 or less and one subject died in the first 72 hours post-exposure. On average per subject, 13.1 (±4.2) tracheobronchial samples were collected during the first 36 hours, and 11.6 (±5.2) blood gas measures were transcribed during the 72 hours of observation.

PaO_2/FIO_2--trend with time

Based on RCM modeling of PaO_2/FIO_2 as a function of time since exposure, there was a small but significant decrease of 1.89 in the ratio of PaO_2/FIO_2 with each cumulative hour up to 72 hours (p=0.001) (Figure 1). PEEP, as a categorical variable (5-9, 10-14, ≥15), was not a significant predictor of PaO_2/FIO_2 over time, nor was percent total body surface burn.

Tracheobronchial inflammatory markers

Log-transformed concentrations of IL-1β, IL-8, and TNF-α were highly correlated, and all three markers showed an increasing trend with time (Figure 2). TGF-β1, in contrast, showed a decreasing trend initially, then leveled off, while sFasL, C5a, and IL-10 showed no apparent trends (Figure 3). Using breakpoint analysis and random coefficient models, IL-1β and -8 increased significantly from zero to 36 hours post-exposure. This increase was steepest during the first 6-7 hours, and plateaued for the remainder of the observation period. Log values of IL-1β increased on average by 0.166 per hour during the first 6.1 hours post exposure (p<0.001) (Figure 4a). Log concentration of IL-8 increased by 0.160

over the first 6.5 hours ($p<0.001$) and by an average of 0.004 per hour between 6.5 and 36 hours ($p=0.398$) (Figure 4b). No significant linear trends over cumulative time were observed for TNF-α, IL-10, TGF-β1, C5a, or sFasL**.**

The effects of initial marker concentrations on the temporal relation between PaO_2/FIO_2 and time since exposure were evaluated in separate random coefficient models in which initial marker concentration was added as a fixed effect to the model. IL-8 was the only marker that was consistently associated with rate of decline in PaO_2/FIO_2. Initial log concentration of IL-8 was associated with an increase in PaO_2/FIO_2 of 47.8 (95% confidence interval, 10.3-85.4, $p=0.013$) over the course of 72 hours (Table 2). Adjusting for PEEP did not improve the fit of these models.

The mean concentrations of inflammatory markers in the initial tracheobronchial sample collected within 6.5 hours of intubation were also evaluated for their relation to subsequent extent of lung injury, either $PaO_2/FIO_2 \leq 200$ within 72 hours of intubation or ARDS (Table 3). Marker concentrations tended to be lower in subjects whose PaO_2/FIO_2 fell below 200. However, only the difference in initial mean IL-8 (log) concentration among subjects with a minimum $PaO_2/FIO_2 \leq 200$ as compared with subjects with a minimum $PaO_2/FIO_2 > 200$ was significant ($p=0.042$). There were no differences in any initial marker concentrations among subjects who developed ARDS and those who did not.

DISCUSSION

In this study of early bronchial inflammatory markers following smoke inhalation in burn victims, there were significant increases in concentrations of IL-1β and IL-8, particularly during the first 6-7 hours after exposure. Other studies have found that increases in proinflammatory cytokines precede development of clinical lung injury, though how early these increases occur has not been established. A number of papers have been published on cytokine profiles over longer intervals of time in ARDS patients,[9,12-16] but none of these papers has been focused on subjects with lung injury resulting from smoke inhalation. The one previously published longitudinal study on a population of severely burned pediatric patients specifically excluded patients with signs of smoke inhalation injury.[17] In the study mentioned, serum cytokines were measured weekly in the burn patients and once in the community controls. Burn patients showed a significant increase in 15 different cytokines during the first week, which declined to the levels of normal patients after five weeks.[17] In cross-sectional studies, higher concentrations of inflammatory mediators have been reported in smoke-exposed patients,[18] in animals exposed to smoke,[3] and in patients with ARDS,[14,19,20] compared with control groups.

Smoke inhalation injury generally presents with severe upper airway injury within 24 hours, while alveolar edema often takes two or three days to manifest.[21] Hence, for this specific type of exposure, collection of tracheobronchial samples may yield the best early markers of lung injury. Bronchoalveolar lavage (BAL) has generally been used to collect samples to evaluate causes of ARDS, which involves changes predominantly at the alveolar level. Several studies, however, have compared the use of BAL to less invasive, nonbronchoscopic techniques for diagnosis of ARDS[22] and of ventilator-associated pneumonia.[23] Though use of these less invasive procedures for diagnosis is not standardized and has not been validated,[23] nonbronchoscopic methods are safer and appear to be associated with fewer physiological derangements.[22] Furthermore, tracheobronchial suctioning is part of standard pulmonary care for smoke inhalation victims and can be repeated frequently. Perkins et al.[24] found no significant difference in mean total protein or protein permeability in samples collected by nonbronchoscopic and bronchoscopic techniques. However, bronchoscopic BAL samples enabled differentiation between

patients at risk of ARDS and patients with ARDS, while nonbronchoscopic samples did not.[24] Unfortunately, no similar studies are available evaluating bronchoscopic vs. nonbronchoscopic sampling of smoke exposure victims.

The increase in IL-1β that we report occurs mostly very early (within 6.1 hours) in response to smoke inhalation injury. Other studies have reported an association between increased mean plasma concentrations of IL-1β on day 1 of ARDS and patient outcome (survival),[12] and increased concentrations of IL-1β in BAL fluid (but not in plasma) and risk of ARDS in trauma, sepsis and shock patients.[9] IL-1ß, secreted by epithelial cells, macrophages and monocytes, increases recruitment of neutrophils and other inflammatory cells and stimulates the secretion of other pro-inflammatory cytokines.[25,26] In rats, intratracheal delivery of IL-1ß resulted in a rapid development of pulmonary neutrophilia and neutrophil-dependent increased lung permeability with edema formation.[26] In the normal physiological state, IL-1ß activity is counter-regulated by the naturally occurring interleukin-1 receptor antagonist and by a circulating IL-1 receptor,[19] which were not measured in this study.

IL-8 increased sharply in the first 6.5 hours after smoke inhalation. Produced by epithelial cells and macrophages, IL-8 is a potent neutrophil chemoattractant. Neutrophilic activity in BAL is thought to be primarily due to IL-8, though there appears to be poor correlation between IL-8 and neutrophils at the onset of ARDS.[19] Although IL-8, measured in the first 24 hours after onset of risk, is reported not to be a predictor of the onset of ARDS, there have been a number of studies that report increased risk of ARDS and mortality associated with high IL-8 levels in BAL or plasma at the beginning of ARDS.[12,16,27-28] In a rabbit model, intravenous treatment with a monoclonal anti-IL-8 antibody prevented smoke-induced changes in alveolar epithelium and vascular endothelium.[3] It may be, however, that the concentration of anti-IL-8:IL-8 complexes will prove to be a better predictor of development of ARDS than IL-8 itself.[29]

Contrary to expectations, in this exploratory analysis of a small number of subjects, initial higher tracheobronchial IL-8 concentrations were correlated with a lesser extent of lung injury. There is no *a-priori* rationale for this finding. One possible interpretation is that sputum IL-8 is protective against lung injury, although this is not consistent with the conclusions of the other studies described above. Alternatively, unmeasured inhibitors of IL-8, such as alpha-2 macroglobulin and serum-derived IL-8 autoantibodies, may increase more than IL-8 in airway suction specimens, resulting in alterations in IL-8 immunoreactivity as well as in its biological effect in certain patients. Finally, IL-8 protein may be modified in those patients with more severe smoke exposures, resulting, through decreased antibody binding, in reduced IL-8 concentration as measured by ELISA.

TNF-α concentrations in the tracheobronchial fluid samples of smoke inhalation victims in our study showed a non-significant increase over time since exposure. TNF-α is thought to directly mediate transcapillary fluid leakage[30] and to promote the adherence of neutrophils to endothelial cells.[31] Although TNF-α levels in BAL fluid of ARDS patients have been shown to be elevated in the early phases of ARDS,[9;12;32] BAL fluid and pulmonary edema fluid levels of TNF-α have not been consistently correlated with clinical outcomes in ALI.[20,33-34] Lymph accumulation of TNF-α was also not involved in smoke-induced ALI in sheep.[6] However, antibodies to TNF-α have been shown to limit septic shock-associated ARDS.[35]

Less research has been conducted on the other inflammatory markers measured in this study. Murine studies indicate an early increase in TGF-β1 in response to bleomycin-induced ALI/ARDS, and a role in increasing permeability of pulmonary epithelial cells mediated via integrin pathways.[36,37] C5a and sFasL have not been studied in association with smoke inhalation injury. However, C5a and sFasL have been detected in the bronchial lavage fluid of ARDS patients and the concentration

of sFasL at the onset of ARDS was shown to be significantly higher in patients who died.[38,39] No studies of IL-10 levels in smoke inhalation victims with ARDS were found; however decreased levels of sputum IL-10 are associated with low-level smoke exposure.[40] Plasma IL-10 levels did not predict the development of ARDS, but were increased in at-risk patients and were significantly higher in ARDS patients who died compared with survivors.[41] However, Armstrong and Millar reported that IL-10 concentrations in the plasma and BAL fluid of patients with ARDS were significantly lower and the ratio of TNF-α/IL-10 in BAL fluid was significantly higher than in at-risk patients.[42]

There are a number of limitations to our study. The number of subjects was relatively small, and information on the extent of smoke inhalation exposure was not available. While not clinically apparent, we were not able to definitively rule out cardiac failure in all patients, so strict adherence to ARDS criteria was not possible. Furthermore, ABGs and chest x-rays were taken based on clinical needs, rather than research needs, so precise determination of time to development of ARDS was not possible. Due to initial methodological problems, early neutrophil counts were available for less than half of the subjects. Thus, we could not ascribe changes in cytokines to cell type. We did not evaluate outcome past 72 hours. Given the severity of smoke inhalation and generally high percentage of total body surface area burned, the results of this study may not be generalizable to smoke inhalation patients treated outside of a burn center. The freezing of samples and subsequent thawing prior to centrifugation to remove cells may have increased the measurable concentration of extracellular markers, and freeze-thawing itself can affect biomarker measurement.[43,44] Although DTT may interfere with some inflammatory marker measurements,[45] use of DTT to homogenize sputum samples is standard protocol. Because all samples were treated similarly, this potential for interference should be minimal and non-differential in nature. In addition, other researchers have not reported an effect of DTT on levels of IL-8 and IL-1β in sputum samples.[46,47] Another potential limitation or direction for future investigations is to look at marker activity levels or the ratio of cytokines with their inhibitors, such as IL-1β/IL-1RA and IL-8/anti-IL-8,[29,33]

which might provide better measures of overall pro- or anti-inflammatory effect. Lastly, because we tested multiple hypotheses related to inflammatory markers and multiple outcomes, it is possible that some of our findings were significant due to chance.

In conclusion, measurement of tracheobronchial secretions provides a means of following inflammatory changes in the airway over time following inhalation injury. We have confirmed that there is a marked temporal increase in concentrations of the pro-inflammatory cytokines IL-1β and IL-8 following smoke exposure, and extended prior observations to demonstrate that this increase occurs in the first 6 hours after smoke inhalation. Additional research is needed to evaluate the prognostic value of the ratios of the selected markers with their inhibitors.

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Table 1. Characteristics of consented subjects with tracheobronchial samples collected within 6.5 hours of intubation (total n= 21)

Mean age (range)	40.8 yrs (12.6-70.1)
Male	20 (95%)
Total body surface area burn	
<15%	8 (38.1%)
15-35%	4 (19.0%)
>35%	9 (42.9%)
Fracture and/or trauma	5 (23.8%)
$PaO_2/FIO_2 \leq 200$ before 72 hrs	14 (66.7%)
Maximum PEEP setting	
5-9	8 (38.1%)
10-14	11 (52.4%)
≥ 15	2 (9.5%)
Died within 72 hrs	1 (4.8%)

Table 2. Random coefficients models predicting PaO_2/FIO_2 over 72 hours, adjusted for initial log concentration of IL-8 (covariance matrix pattern for random effects unstructured).

	Estimate	**SE**	**p-value**
Fixed-effects Parameters			
intercept	132.57	81.65	0.104
cumulative hours	-1.94	0.57	0.001
(log) initial IL-8	47.83	19.17	0.013
Random–effects Parameters			
sd (intercept)	88.61	22.39	
sd (slope)	2.00	0.58	
correlation (intercept, slope)	-0.85	0.11	
sd (residual)	99.93	5.11	
Log likelihood	-1416.7		

Table 3. Median (interquartile range) of initial marker concentrations by subsequent outcome.

	*$PaO_2/FIO_2 \leq 200$ (n=14)	*$PaO_2/FIO_2 > 200$ (n=7)	ARDS (n=9)	no ARDS (n=12)
IL-1β (pg/ml)	697 (249, 1481)	2064 (236, 8694)	988 (257, 1481)	804 (210, 2698)
IL-8 (pg/ml)	10380 (2773, 14072)	57566 † (10694, 76756)	10893 (2966, 14072)	16175 (6010, 61000)
IL-10 (pg/ml)	3.0 (0.5, 5.3)	3.2 (0.5, 18)	3.1 (0.5, 5.3)	3.1 (0.5, 12)
TNF-α (pg/ml)	68 (17, 215)	208 (30, 1096)	64 (18.8, 215)	136 (24, 768)
TGF-β1 (pg/ml)	112 (9.8, 270)	200 (11.4, 576)	152 (106, 361)	61 (9.8, 340)
C5a (pg/ml)	0.18 (0.18, 96)	11 (1.85, 307)	0.18 (0.18, 281)	3.8 (0.18, 95)
sFasL (pg/ml)	10.1 (1.0, 35)	14.7 (1.0, 46)	21.2 (1.0, 35)	1.1 (1.0, 40)

***** Lowest PaO_2/FIO_2 at any time during the 72 hour observation period

† p =0.042, 2-sided t-test using log-transformed values, unequal variances assumed

FIGURE LEGENDS:

Figure 1. Relationship between PaO_2/FIO_2 and cumulative time since smoke exposure, based on fitted regression lines from a random coefficients model.

72 hour model: Predicted=326.8 – 1.89(hours) + ε

Figure 2. Mean concentration of log of IL-8, IL-1β, and TNF-α (pg/ml) in tracheobronchial fluid samples collected between intubation and 36 hours post-intubation.

Figure 3. Mean concentration of log of TGF-β1 (pg/ml), IL-10 (pg/ml), sFasL (pg/ml), and C5a (ng/ml) in tracheobronchial fluid samples collected between intubation and 36 hours post-intubation.

Figure 4. Relation of IL-1β and IL-8 over time since smoke inhalation. Breakpoint analysis and random coefficient modeling were used to fit two regression lines to each model, constrained to join at a common point. a) Log concentration of IL-1β increased by an average of 0.166 per hour over the first 6.1 hours, and declined by 0.0005 per hour thereafter; b) log concentration of IL-8 increased by an average of 0.160 per hour over the first 6.5 hours and 0.004 per hour between 6.5 and 36 hours.

Figure 1.

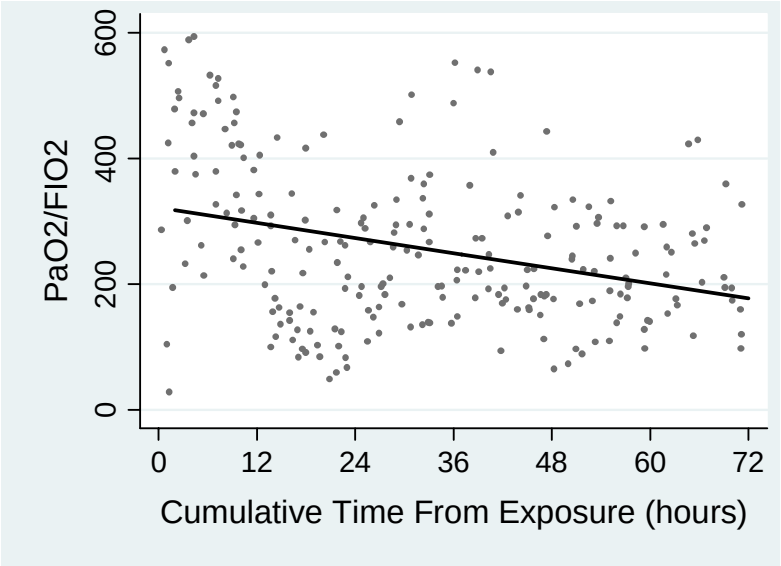

PaO2/FIO2
0
200
400
600
0
12
24
36
48
60
72
Cumulative Time From Exposure (hours)

Figure 2.

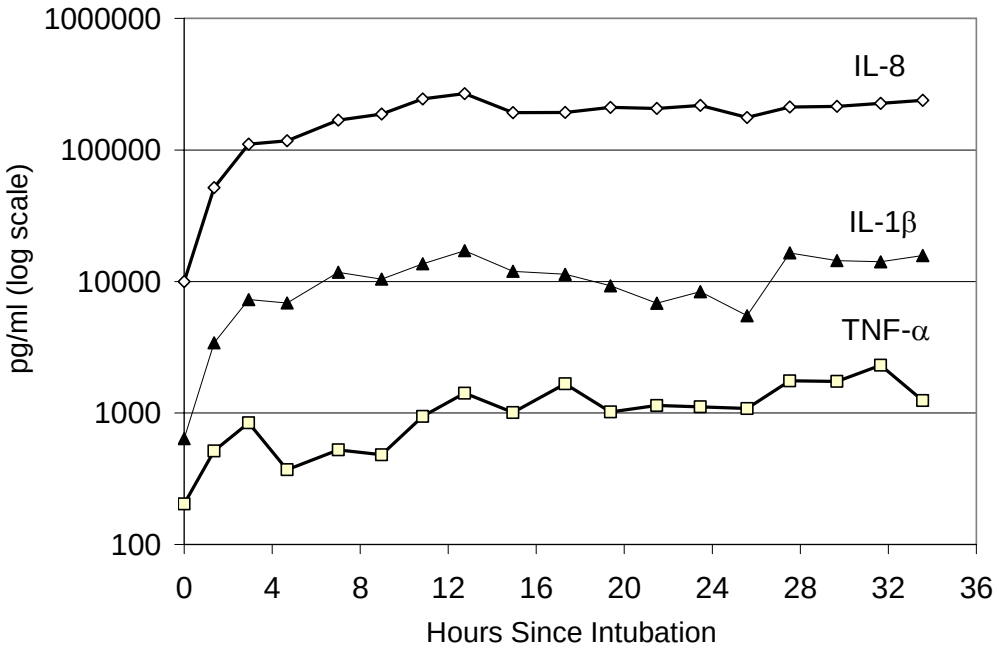

1000000
100000
10000
1000
100
pg/ml (log scale)
IL-8
IL-1β
TNF-α
0
4
8
12
16
20
24
28
32
36
Hours Since Intubation

Figure 3.

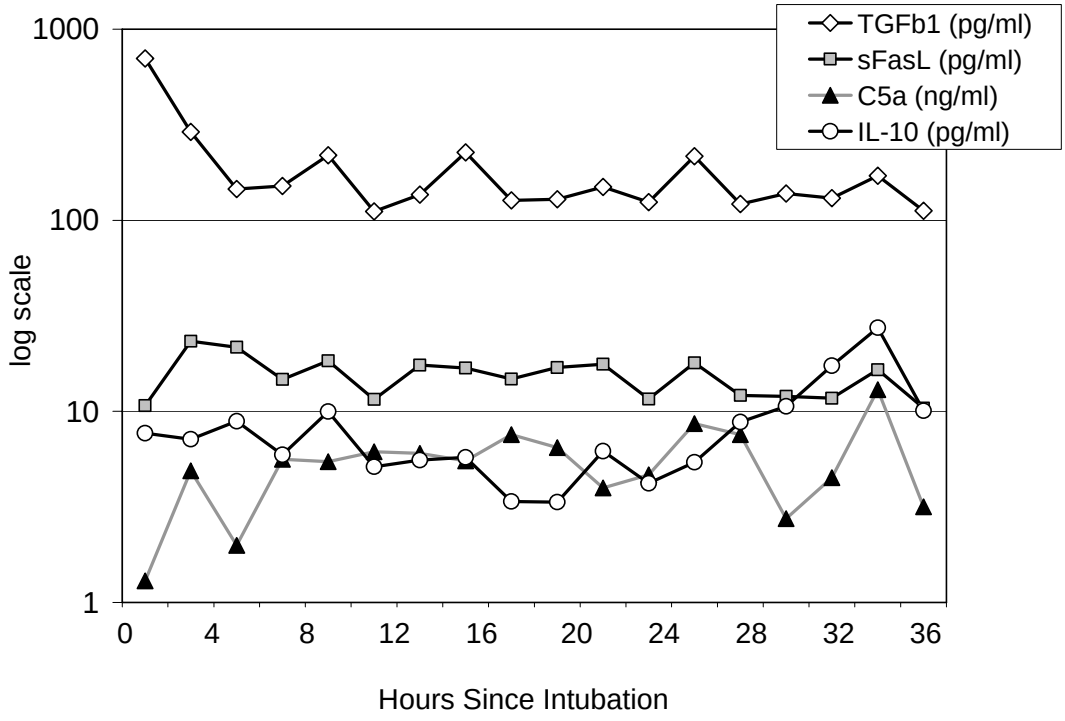
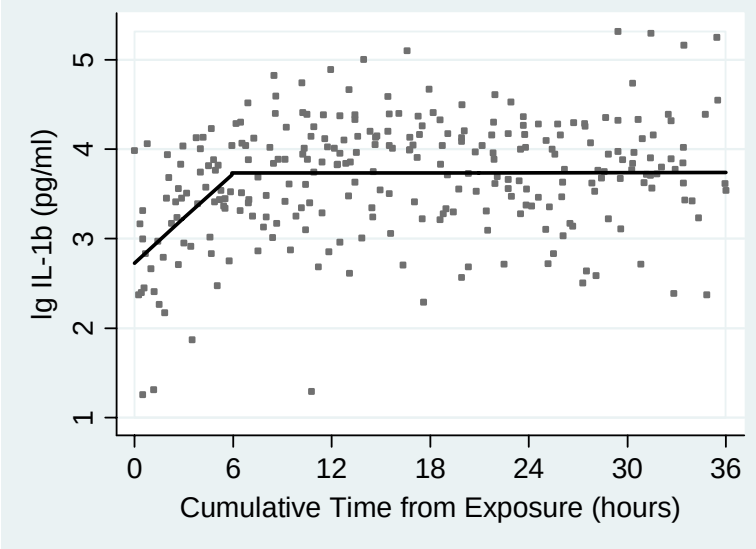
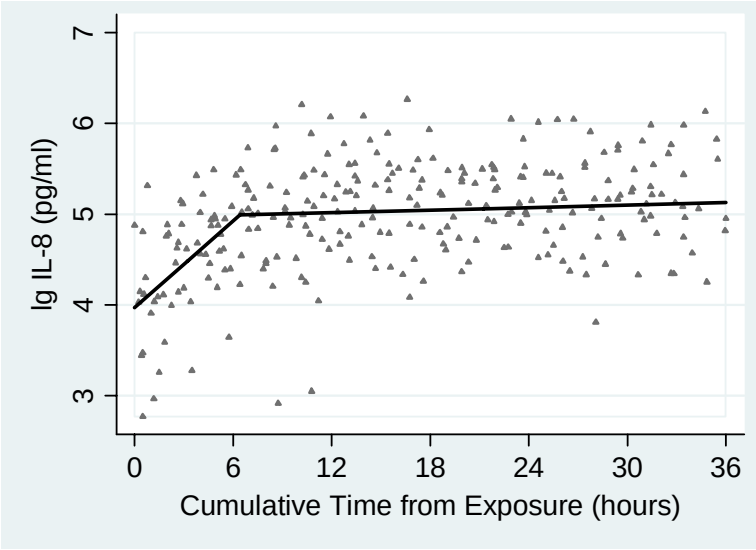

TGFb1 (pg/ml)
sFasL (pg/ml)
C5a (ng/ml)
IL-10 (pg/ml)
1000
100
10
1
log scale
0
4
8
12
16
20
24
28
32
36
Hours Since Intubation

Figure 4.

a)


lg IL-1b (pg/ml)
1
2
3
4
5
0
6
12
18
24
30
36
Cumulative Time from Exposure (hours)

b)


lg IL-8 (pg/ml)
3
4
5
6
7
0
6
12
18
24
30
36
Cumulative Time from Exposure (hours)